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Clinical and molecular features associated with biallelic mutations in *FANCD1/BRCA2*

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Abstract

Patients with biallelic mutations in *BRCA2* are classified as Fanconi Anemia (FA) complementation group D1. We analyzed the severity of the *BRCA2* mutations in 26 cases identified from the literature and one from our National Cancer Institute Cohort. Mutations were classified according to their association with breast cancer in heterozygotes reported to the Breast Cancer Information Core, as well as the predicted functional impact of the mutations. Twenty mutations were frameshifts or truncations, three involved splice sites, five were missense variants of unknown clinical severity, and two were benign polymorphisms. Five patients had VATER association. Leukemia was reported in 13 patients, and solid tumors in 15; 6 patients had two or more malignancies. Only 2 patients had no cancer at ages 2 and 30 years. The cumulative probability of any malignancy was 97% by age 5.2 years. IVS7+1G>A and IVS7+2T>G were associated with AML, and 886delGT and 6174delT with brain tumors. However, patients with other alleles remained at very high risk of these events compared with patients from other FA complementation groups. Missense mutations formed a distinct cluster in a highly-conserved region of the BRCA2 protein between residues 2236 and 2729.

The small group of patients with biallelic mutations in *BRCA2* is distinctive in the severity of the physical phenotype, as well as early onset and high rates of leukemia and specific solid tumors, and may comprise an extreme variant of FA. While half of the families had relatives with *BRCA2*-type cancers, several of the alleles reported in probands were not associated with cancer in presumed carriers, and thus counseling presents more uncertainties than usual.

Fanconi Anemia (FA) is a genetically heterogeneous and predominantly autosomal recessive disorder characterized by defective DNA repair with high rates of birth defects, aplastic anemia, leukemia, and solid tumors [1]. FA is associated with homozygous or biallelic mutations in at least twelve genes, *FANCA*, *FANCB* (X-linked recessive), *FANCC*, *FANCD1*, *FANCD2*, *FANCE*, *FANCF*, *FANCG*, *FANCI* (not yet cloned), *FANCJ*, *FANCL*, and *FANCM* [2]. In response to DNA damage, the protein products of all of these genes (except for *FANCD1*, *FANCD2* and *FANCJ*) form a complex, which is required for the ubiquitination of the protein product of *FANCD2*, a key component of the DNA damage repair pathway [3].

More than 1000 unique mutations in *BRCA1* or *BRCA2* [4] are associated with familial breast/ovarian cancer [5]. The molecular bases of these seemingly different genetic disorders converged with detection of BRCA1 proteins in FA-related DNA damage-response foci [6], and in the discovery of biallelic mutations in *BRCA2* in patients classified as FA complementation group FA-D1 [7]. Furthermore, patients with an FA clinical phenotype were recently found to have germline mutations in two genes which act downstream of the D2 ubiquitination step, namely *BRCA2*, and *FANCJ/BRIP1/BACH1*, the latter a gene whose product may interact with BRCA1 [8, 9]. The precise roles of these downstream genes in FA have not been entirely defined.

In view of the mechanistic implications of this nexus between two distinct cancer susceptibility syndromes and molecular pathways, we analyzed the clinical and molecular features associated with the *BRCA2* mutations identified in patients classified as FA-D1, using data from 26 previously-reported cases and findings from a newly-diagnosed case. Clinically, we noted that several patients had physical anomalies consistent with the VATER association and its variants (MIM 192350 and MIM 276950 [10]), and we evaluated genotype/cancer correlations between specific mutations in *BRCA2* and specific types of malignancies. Homozygous mutations in *BRCA2* are embryonic lethal in mice, and it had been assumed that humans carrying homozygous mutations would also fail to come to term [11, 12]. Therefore, we evaluated whether the reported *FANCD1/BRCA2* mutations led to functional inactivation of the *BRCA2* gene or a physiologically meaningful diminution of the *BRCA2* gene product. Finally, we mapped the intra-genic location of each of the FA-related *BRCA2* mutations, and quantitatively compared this distribution to that among heterozygotes reported to the Breast Cancer Information Core (BIC) database (http://research.nhgri.nih.gov/bic/) [13].

Subjects and Methods

We identified published cases with biallelic mutations in *FANCD1/BRCA2* from a PubMed search using combinations of the terms “Fanconi Anemia”, “FANCD1”, “BRCA2”, and “VATER.” We diagnosed a previously-unreported case in our NCI Inherited Bone Marrow Failure Syndromes Cohort [14]. The protocol for this cohort was approved by the Institutional Review Board of the National Cancer Institute.

Analysis of BRCA2 Alleles

Information regarding the functional consequences of each allele reported in FA-D1 patients with regard to breast cancer was obtained from the Breast Cancer Information Core (BIC) database [13]. Mutations were classified as “deleterious” (truncating or splice-site mutations predicted to disrupt the function of the protein, which are associated with a greatly-

increased risk of breast cancer), “probably deleterious” (likely to disrupt normal splicing), “unknown” or “unclassified variants” (missense mutations of unknown clinical significance with regard to breast cancer risk), or "benign" (not associated with increased risk of breast or ovarian cancer). We then compared the phenotypes of the patients with the prior classification of the severity of the mutations. We were surprised to find that five of the FA-related *BRCA2* mutations were categorized as unclassified variants, and two were benign polymorphisms. Alleles classified as benign polymorphisms are commonly found in control populations and have not been associated with breast cancer in case/control studies. Since the mutation data for this review were derived from the literature, we cannot exclude the possibility of additional undiscovered deleterious mutations in *cis* with those reported, such as large deletions, which might lead to reclassification of the severity of the mutations which we have called “benign” or “of unknown significance”.

Positional Analysis

Positional analysis considered whether the FA-associated missense mutations had an “unusual” distribution along the protein sequence, as described below. As a reference, we considered the location of all unclassified *BRCA2* missense mutations reported to the BIC. We asked whether the FA-associated missense mutations were distributed uniformly, non-uniformly but with the same frequency curve as other missense mutations in the BIC, or non-randomly (i.e. in a cluster). We employed exact and non-parametric statistical tests because of the small sample size.

The BRCA2 protein contains 3418 amino acids. If the n= 5 FA-related missense mutations were distributed randomly, then $1/3$ each should fall in positions 1:1139, 1140:2279, and 2280:3418. Under this hypothesis, the observed frequency counts (N_1, N_2, N_3) constitute a sample from a multinomial distribution with equal frequencies, and the Pearson Chi-Square statistic $\sum_{i=1}^{3}(N_i - n\frac{1}{3})^2/(n\frac{1}{3})$ provides a measure of the discrepancy between observed and expected values. We computed the exact distribution of this statistic under the null hypothesis and used this distribution to obtain an exact p-value.

To determine whether FA missense mutations have the same distribution as other missense mutations reported to the BIC, we used a Wilcoxon rank sum test to compare the median position of FA missense mutations with the median position of 733 distinct *BRCA2* missense mutations found in the BIC. To specifically test for a ‘cluster,’ we considered the distance between the most NH2 and - COOH proximal positions of the FA missense mutations, i.e., the observed range statistic, compared with the expected distribution of the range statistic computed from 100,000 Monte Carlo simulations. The reported p-value equals the probability of observing a range statistic as small as, or smaller than, what was observed. The expected distribution of the range was computed two ways, assuming the positions of the FA missense mutations were a sample from a uniform distribution, and sampling 5 mutations with replacement from the 733 BIC missense mutations.

We performed similar analyses of the FA-associated truncation or frameshift mutations.

Genotype/Phenotype Correlations

The correlation between the presence of a specific mutation and a specific outcome was assessed using the odds ratio (OR) and its 95% confidence interval (CI); significance was tested with the Fisher exact test. The relative hazard of an event in those with or without the mutation was estimated using the Cox proportional hazards model. Statistical analyses were performed using Stata9 [15].

Cancer History in Families

Data on the types and ages of cancer in family members were obtained from the published case reports, and from the pedigree obtained by interviewing family members of our new case.

Results

Case Report

A 2100 gm 37 week female (NCI 1) had been identified *in utero* with hydrocephalus, fused kidneys, and growth retardation. At birth, she had intrauterine growth retardation, corneal opacities (diagnosed as Peters anomaly, MIM 604229), an anteriorly-placed anus, small kidneys, and long thumbs with increased laxity; this constellation led to a later diagnosis of VACTERL-H (at least three of: vertebral anomalies, anal atresia, cardiac anomalies, tracheoesophageal fistula, esophageal atresia, radial limb anomalies, renal anomalies, plus hydrocephalus, MIM 276950). Other physical findings included microcephaly, facial dysmorphia, microphthalmia, esoptropia, growth failure, café-au-lait spots, and malposition of the kidneys. Surgical procedures during the first year included a ventriculoperitoneal shunt, anoplasty, and repair of a tethered spinal cord due to a filum terminale lipoma.

Her karyotype was 46,XX, with “structural chromosome changes that may reflect chromosome instability, an acute viral infection, or artifact of culture.” The chromosome breakage test for FA was not done until age 20 months, when an astute geneticist observed her birth defects and suggested the diagnosis of FA. Chromosome breakage studies of peripheral blood lymphocytes with both diepoxybutane and mitomycin C were diagnostic of FA. Direct DNA sequencing (Myriad Genetics, Inc.) revealed biallelic mutations in *BRCA2*, 6174delT and Q3066X, thereby identifying her as belonging to group FA-D1. At age 3.1 she was diagnosed with a medulloblastoma.

Genotype/Birth Defect Associations in Patients with BRCA2 Mutations

The published cases and our new case are summarized in Table 1 [7, 16-25]. The series includes 27 patients from 21 families. One family was not genotyped, and there were 30 unique alleles among the 40 alleles that were typed. Twenty of the FA-related *BRCA2* mutations were were frameshift or truncating mutations three involved splice sites, five were missense variants of unknown clinical severity, and two were benign polymorphisms. Pathogenicity of the specific mutations is described below.

Table 1: Patient characteristics, malignancies, and BRCA2 Mutations

Pt ID	Phenotype	Sex	Cancer (age, yrs)	Allele 1[b]	Allele 2[b]	Interpretation 1	Interpretation 2	Reference
HSC62	Abnormal thumb	M	None (30)	IVS19-1 G>A	IVS19-1 G>A	Probably deleterious	Probably deleterious	[7]
EUFA423	Pigmented, abnormal thumb	F	Brain (3)	7691/insAT [R2488fs]	9900/insA	Deleterious	Deleterious	
HSC230	Pigmented, abnormal thumb	M	None (2)	3033/delAAAC	10204A>T [K3326X]	Deleterious	Benign	
EUFA579	Pigmented, abnormal thumb	F	AML	7235G>A [R2336H][c]	5837TC>AG [F1870X]	Unknown	Deleterious	
AP37P	Short, pigmented, café au lait, abnormal thumb, Sprengel, midface hypoplasia	M	AML M2 (2)	8415G>T [K2729N]	8732C>A [S2835X]	Unknown	Deleterious	[7, 17]
1A	Short, café-au-lait, pigmented, abnormal thumbs and radii, microcephaly, imperforate anus, epicanthal folds, micropenis, undescended testes, dislocated hips, hydronephrosis, abnormal hearing (VATER[a])	M	Brain – medulloblastoma or astrocytoma (4.9)	6174delT	9435T>A [C3069X]	Deleterious	Deleterious	[18, 19]
1B	Short, abnormal thumbs, microcephaly, imperforate anus with rectovaginal fistula, slanted eyes, anomalous kidneys, small ear, hip dysplasia (VATER[a])	F	Brain - astrocytoma (2)	6174delT	9435T>A [C3069X]	Deleterious	Deleterious	
2	No exam reported	F	Brain – medulloblastoma (4.5)	6174delT	886delGT	Deleterious	Deleterious	[18]
3	No exam reported	F	Brain – medulloblastoma (2.5)	5301insA	7690T>C [I2490T]	Deleterious	Unknown	
4	No exam reported	F	Brain – medulloblastoma (3.5)	4150G>T [E1308X]	9424C>T [Q3066X]	Deleterious	Deleterious	
K1S1	Café au lait, microcephaly, cardiac	M	Brain – medulloblastoma (2.3)	886delGT	8447T>A [L2740X]	Deleterious	Deleterious	[20]

K1S2	Café au lait, abnormal facies, epicanthus	M	Wilms (1.3), Brain – medulloblastoma (4.3)	886delGT	8447T>A [L2740X]	Deleterious	Deleterious	
K2S1	Short, microcephaly	M	Wilms (0.5), AML (2)	4876G>T [E1550X]	7757T>C [L2510P]	Deleterious	Unknown	
K2S2	Short, pigmented, bifid thumb, elfin facies, small palpebral fissures	F	T-ALL (4.9)	4876G>T [E1550X]	7757T>C [L2510P]	Deleterious	Unknown	
129/1	Short, café au lait, microcephaly, imperforate anus	N/A	AML (2.2)	IVS7+2T>G	IVS7+2T>G	Probably deleterious	Probably deleterious	[21, 22]
357/1	Short, thumb, imperforate anus	N/A	AML (1.9)	8106G>C [W2626C]	2041insA	Unknown	Deleterious	
632/1	Short, café au lait, dysplastic hips, pelvic kidney	F	AML (3)	IVS7+1G>A	5910C>G [Y1894X]	Probably deleterious	Deleterious	
632/2	Short, imperforate anus, hypoplastic thumb	F	AML (1.8)	IVS7+1G>A	5910C>G [Y1894X]	Probably deleterious	Deleterious	
800/1	Short, café au lait, microcephaly, micropenis	M	AML (0.9)	IVS7+2T>G	5164del4	Probably deleterious	Deleterious	
800/2	Short, café au lait, microcephaly	M	Wilms (0.8)	IVS7+2T>G	5164del4	Probably deleterious	Deleterious	
900/1	Short, café au lait	M	T-ALL (5.2)	2816insA	1342C>A [N372H]	Deleterious	Benign	
984/2	Short, microcephaly, hypoplastic thumb, labial adhesions, patent foramen ovale, CNS venous anomaly	F	T-ALL (4.9), AML (6.3), Wilms (6.6)	N/A	N/A	N/A	N/A	
RB	Short, pigmented, café au lait, microcephaly, cryptorchidism	M	Wilms (3.5), Brain – glioblastoma multiforme (9)	886delTG	5873C>A [S1882X]	Deleterious	Deleterious	[22]
CB	Pigmented, café au lait	M	Wilms (0.6), Brain – medulloblastoma (6), B-ALL (10)	886DelTG	5873C>A [S1882X]	Deleterious	Deleterious	
SB1690CB	Hypermobile thumb, microcephaly, imperforate anus, deaf, renal dysplasia, midfacial hypoplasia (VATER[a])	M	AML (2.1)	IVS7+2T>G	3827delGT	Probably deleterious	Deleterious	[24]
PT2	Short, pigmented, café au lait,	F	Wilms (1),	1548del4	1548del4	Deleterious	Deleterious	[25]

	adducted thumbs, microcephaly, sacral hemivertebra, ventricular septal defect, pelvic kidney, esophageal atresia, micrognathia, CNS gyrations, congenital cataract (VATER[a])		Neuroblastoma (1.1), Brain – posterior fossa (3)					
NCI 1	Short, café au lait, microcephaly, facial dysmorphia, abnormal thumbs, anterior anus, cloudy corneas, ectopic kidneys, delayed development, hydrocephaly (VATER[a])	F	Brain - medulloblastoma (3.1)	6174delT	9424C>T [Q3066X]	Deleterious	Deleterious	Current report

1A and 1B were second cousins once removed; K1S1 - K1S2, K2S1 - K2S2, 632/1 - 632/2, 800/1 - 800/2, and RB - CB were sib pairs.

N/A, not available

[a]VATER association includes vertebral anomalies, anal atresia, ,tracheoesophageal fistula with esophageal atresia, and abnormal radii. VACTERL adds cardiac, renal, and limb anomalies. VACTERL-H includes hydrocephalus. In cases without VATER there may have been incomplete reporting of the physical features.

[b]Alleles are shown as nucleotide changes. Where the change results in a missense or nonsense mutation, the resulting [amino acid change] is indicated below the nucleotide.

[c]R2336H (7235 G>A) is classified as a missense mutation. It alters the last "G" nucleotide in exon 13 of *BRCA2*. In addition to altering this codon, mutation of this G to A could alter splicing.

Several recurrent birth defects were reported, including hyperpigmentation and/or café-au-lait spots, short stature, microcephaly, abnormal thumbs, and renal anomalies. Although small numbers make it difficult to judge whether these features occurred more frequently than expected, five patients (1A, 1B, SB1690CB, PT2, and NCI 1) had three or more of the VATER association anomalies. In these patients, both mutations in *BRCA2* were considered to be deleterious or probably deleterious (see below). We detected no significant association between specific *BRCA2* mutations and VATER association. Among the five patients with VATER association two cousins had brain tumors (cousins 1A and 1B); one had AML (SB1690CB); another had Wilms tumor, neuroblastoma, and brain tumor (PT2); and NCI 1 had a brain tumor at age 3.1 years. The distribution of tumor types did not differ between the patients with and without VATER features.

Two patients (HSC62 and PT2) carried homozygous mutations that are probably deleterious or deleterious, IVS19-1 G>A and 1548del4 respectively, both of whom had birth defects. The homozygous IVS19-1 G>A patient had no tumor by age 30; the homozygous 1548del4 patient had VATER features, Wilms tumor, neuroblastoma, and brain tumor, all by age 3 years.

The spectrum of physical findings reported in all five of the patients with one allele of unknown clinical significance appeared to be similar to those in patients with two deleterious alleles; it is of interest (but not statistically significant) that none of these patients met criteria for VATER association.

Genotype/Cancer Associations in FANCD1/BRCA2 Patients

The types and ages of cancers reported in FA-D1 patients are summarized in Table 1. Five of the 10 patients with AML had mutations in IVS7 (two were IVS7+1G>A, and three were IVS7+2T>G), and five had a variety of other mutations. One additional patient with IVS7+2T>G died at less than one year of age from stem cell transplant complications, without developing AML. While the OR for the association of AML with IVS7 mutations compared with other *BRCA2* mutations was 15 (95% CI 1.1-750, p = 0.02), patients with other *BRCA2* mutations also had a high risk of AML [26]. The OR for AML in 10 of 27 FA-D1 patients compared with 9 of 145 FA patients (all other complementation groups combined) whose mutations were not classified [27] is 9 (95% CI 2.8-28, p <0.00001).

Reid *et al*. suggested a correlation between *BRCA2* 886delGT and brain tumors [23]. In our review, we found 12 patients who had brain tumors, five of whom had one 886delGT allele, an allele observed only in brain tumor patients (OR ∞, p = 0.006). The 12 patients with brain tumors were from nine families and included three pairs of siblings, and only three of the nine families had the 886delGT allele. Thus, the observed association might be due to other familial risk factors for brain tumors, since the number of families is small. Seven patients with brain tumors from six families did not carry 886delGT, indicating that patients with other mutations are also at risk of brain tumors. For example, 6174delT occurred in four patients, all of whom had brain tumors (OR ∞, p = 0.03). One patient had both 886delGT and 6174delT. Seven of the brain tumors were medulloblastomas, and the others were described respectively as either medulloblastoma or astrocytoma, astrocytoma, glioblastoma multiforme, posterior fossa tumor, and brain tumor.

Wilms tumor was reported frequently in these patients. Three of the seven cases had the 886delGT mutation (OR 6.8, 95% CI 0.5-98, p = 0.09), two of whom were siblings; the association between this allele and Wilms tumor may be familial rather than *BRCA2*-specific. Comparing the 15 FA-D1 patients who had solid tumors with 14 patients with tumors among 145 unclassified FA patients in our previous report [27] yielded an OR = 12 (95% CI 4-22, p <0.00001) for FA-D1 patients and solid tumors.

Two of the 27 patients had no cancer history at the time they were reported (ages 2 and 30 years): one was homozygous for a probably deleterious allele (HSC62), and one had a benign *BRCA2* allele plus a mutation in *FANCB* (HSC230) (see below). The other patient with one benign *BRCA2* allele had T-cell ALL, which is atypical for FA, in which AML is the usual subtype. The OR for any malignancy in 25 patients with FA-D1 compared with 23 of 145 FA patients in our earlier study [27] was 66 (95% CI 14-594, p <0.00001).

In the current series, the cumulative probability of developing leukemia was 79% by age 10 [26]; the probability of developing a solid tumor reached a plateau of 67% by age 5 years (Figure 1A). The most common solid malignancy was brain tumor: probability among all patients was 64% by age 5 (Figure 1B). All five patients with the 886delGT allele developed a brain tumor by 4.5 years (median age 2), while seven of the 22 patients with other alleles developed their brain tumors by 4.9 years of age (Figure 1C). The age at diagnosis of a brain tumor was significantly earlier in those with mutations in 886delGT (p = 0.02 by Cox regression). The cumulative probability of developing a Wilms tumor was 64% by age 6.7 (data not shown), and of any malignancy was 97% by age 5.3 years (Figure 1D).

Severity of BRCA2 Mutations in Patients

Twenty of the 30 unique alleles among the 40 alleles were classified as “deleterious”, based on reports in the BIC database and on their structure (nonsense or frame-shift mutations leading to early protein truncation, Table 1). Three alleles which involve splice sites (IVS19-1 G>A, IVS7+2T>G, and IVS7+1G>A) were designated as “probably deleterious.” According to the BIC database, both mutations in IVS7 in *BRCA2* have been found in families with breast and ovarian cancer. These mutations alter a splice site which results in skipping of exon 7. In one patient with breast cancer and the IVS7 +2T>G mutation, no RNA was produced *in vitro* from the chromosome carrying the mutation [28]. Five missense mutations were of “uncertain significance,” since they were not reported to be significantly associated with breast or ovarian cancer in the BIC.

In addition, in two patients one allele was classified as a benign polymorphism by the BIC. K3326X in patient HSC230 is known to be a polymorphic stop codon [29]. This patient was originally and correctly classified as FA-B, not FA-D1, and had a frameshift mutation in exon 8 of *FANCB* (1838insT) which leads to a premature stop codon [16]. He had been screened for mutations in *BRCA2* before the *FANCB* gene was identified. To complicate matters further, HSC230 was recently found to be a *BRCA2* mosaic: fibroblasts heterozygous for 3033/delAAC and wild type *BRCA2*, and lymphoblasts that contain both mutant *BRCA2* alleles [30]. Thus, the FA phenotype of HSC230 is due to the mutation in *FANCB*, and not to the combination of a deleterious mutation and a benign polymorphism in *BRCA2*. In fact, this patient had not

developed cancer when reported at age 2. We have retained this patient on our list of "*FANCD1/BRCA2*" subjects in order to emphasize the caution required in interpretation of the causal role of mutations in *BRCA2* in FA.

Case 900/1 also had both a deleterious allele and a benign polymorphism (N372H) in *BRCA2*, and was reported to have a mild phenotype, with café-au-lait spots and short stature. The N372H allele was not associated with increased sporadic breast cancer risk in the Nurses' Health Study cohort [31]. This male patient was reported not to belong to FA groups A, C, D2, E, F, or G, but was apparently not tested for B, L, M or J, and thus classification of this case as FA-D1 must also remain uncertain.

Severity of BRCA2 Mutations in Families of Patients with FANCD1

Family histories were examined to determine whether the types of cancers seen in breast/ovarian cancer families [5] were also reported in the FA families. This analysis is very limited, as several articles did not mention family history, and the ages of other family members at risk of cancer were not provided. If both *BRCA2* alleles in FA-D1 patients have deleterious mutations, it might be expected that breast and/or ovarian cancer would be a frequent occurrence in *both* parental bloodlines, since both parents of affected children would be obligate carriers of a single copy of a deleterious allele, as would half of each parent's first-degree relatives. While the data set is small and subject to ascertainment bias due to the known association between *BRCA2* and breast/ovarian cancer, it is noteworthy that cancers associated with carriers of mutations in *BRCA2* were reported in 11 of the 21 families (Table 2).

Among the 11 families in which cancer was reported, the breast cancer occurred on the side of the family carrying a "benign" allele (N372H) in family 900/1; in four families cancer was noted on only one side of the family (357/1, 800/1, 900/1 and SB1690CB), in four families the cancer history is not extensive, and in only two families was there a strong family history of breast cancer or breast cancer-associated cancers (1A and 1B, and NCI 1). In five families, breast cancer was reported in both maternal and paternal relatives, while in four there was a breast cancer history on only one side of the family, and in two only non-breast cancers were mentioned, one of which (brain) is not part of the breast/ovarian cancer spectrum (K2, 800/1). Twenty-seven female relatives (13 below age 50) and one male relative had breast cancer. In the parental generation, there were one prostate cancer at age 65 in a carrier of C3069X, and two breast cancers at ages 38 and 45 in carriers of Y1894X and S1882X; these are all known deleterious mutations. At the level of the grandparents of the probands, six grandmothers (out of 22 in the eleven families reported) had breast cancer at ages 29, 40, 46, 48, 65 and 70. The *BRCA2* alleles were not specifically identified in the grandparents, although the daughter of the grandmother with breast cancer at age 29 carried the benign N372H allele. The male breast cancer developed in a grandfather at age 60; his daughter carried the deleterious mutation Y1894X.

Table 2: Cancers in Families of *FANCD1/BRCA2* Probands

Patient ID	Maternal Allele[a]	Maternal Cancers	Paternal Allele[a]	Paternal Cancers	Reference
1A	6174delT	*Breast*: grandaunt (paternal) 76; *Colon*: grandaunt (paternal)	9435T>A [C3069X]	*Breast*: grandmother 65; *Melanoma*: granduncle (maternal)	[17]
1B	6174delT	*Breast*: aunt 58, grandaunt (maternal) 70s; *Breast and lung*: grandaunt (maternal)	9435T>A [C3069X]	*Prostate*: father 65, grandfather 70s	
K1	8447T>A [L2740X]	No information available for maternal family	886-887delGT	*Breast*: great grandmother (paternal) 90s; *Colorectal*: great grandfather (paternal) 50s; *Renal*: grandmother 63; *Skin of lip*: grandfather 50s, *Unknown*: great grandfather (maternal) 80s	[20]
K2	4876G>T[b] [E1550X]	*Colorectal*: great grandmother (maternal) 60s	7757T>C[bd] [L2510P]	*Prostate*: grandfather 70s	
129/1	IVS7+2T>G	*Breast*: aunt 45, *Pancreas*: uncle 51	IVS7+2T>G	*Breast*: grandmother 40	[21]
357/1	2041insA	*Breast*: grandmother 46, great grandmother 46, great grandaunt 45; *Prostate*: grandfather 48; *Bladder*: great grandfather 51	8106G>C [W2626C][c]	No cancer reported	
632/1	5910C>G [Y1894X]	*Breast*: mother 38; *Male breast*: grandfather 60	IVS7+1G>A	*Breast*: grandmother 48	
800/1	5164del4	*Brain*: great grandfather 50	IVS7+2T>G	No cancer reported	
900/1	1342C>A [N372H][c]	*Breast*: grandmother 29	2816insA	No cancer reported	
RB	5873C>A [S1882X]	*Breast*: mother 45	886delTG	*Breast*: aunt 48	[23]
SB1690CB	IVS7+2T>G[b]	*Breast*: aunt 40s, grandmother 70s	3827delGT[a]	No cancer reported	[24]
NCI 1	6174delT	*Breast*: (maternal) grandaunt 42, (paternal) grandaunt 58, 7 other paternal relatives 35, 41, 51, 50s, 50s, 55, age unknown; other paternal relatives with cancer: Males - *Wilms*: 6[d]; *Bladder*: 50s; *Unknown* in 2 individuals; Females - *Leukemia*: 20s; *Uterus*: great grandmother; *Unknown*: 1 individual.	9424C>T [Q3066X]	*Breast*: (maternal) great grandmother 51; *Voice box*: (paternal) great grandfather age unknown	Current report

[a]Maternal and Paternal columns refer to the parents of the proband; (paternal) or (maternal) in parens refer to the lineage in relation to the mother or father. For example, grandaunt (paternal) refers to the mother's father's sister. [b]Reference does not identify segregation of maternal versus paternal alleles. [c]Benign. [d]Unknown. Alleles which are known to be deleterious or probably

deleterious are not indicated with a superscript. Note that cases 1A and 1B are related (see legend to Table 1).

No ovarian cancer was reported, which may not be surprising given the small number of families and the fact that ovarian cancer penetrance is significantly lower in carriers of mutations in *BRCA2* compared with *BRCA1*. There were four cases of prostate cancer, one in the father of a case, and three in grandfathers (one at age 48); prostate cancer is part of the *BRCA2* cancer susceptibility spectrum [5]. There were also three cases of colon cancer, two cases of bladder cancer, one melanoma, and a small number of other, not clearly *BRCA2*-related, cancers. In one family maternal cancer history was not available, and in three other families the paternal family history did not report any cancers (two of these involved deleterious alleles). Although the currently available data are not sufficiently robust to address this question definitively, the family history information does suggest that a single copy of some of the deleterious *BRCA2* alleles which are found in FA-D1 patients confers a pattern of cancer susceptibility similar to that described for this gene in the context of hereditary breast/ovarian cancer. However, the data also suggest that several of the alleles reported in patients with *FANCD1/BRCA2* are not associated with increased rates of cancer in family members who are presumed to be carriers of one of the alleles.

Location of the FANCD1/BRCA2 Mutations

The *FANCD1/BRCA2* mutations were distributed throughout the length of the *BRCA2* gene (Figure 2), and the two benign polymorphisms, N372H and K3326X, were at the N-terminal and C-terminal respectively. However, the FA-associated missense mutation-related amino acid changes were not uniformly distributed across the BRCA2 protein ($p = 0.012$, exact Pearson statistic). The median locations for the 5 FA and 733 BIC-associated missense mutations were dissimilar, but not significantly so (median positions 2510 and 1889, respectively, Wilcoxon $p = 0.10$). However, the FA-associated missense mutations were tightly clustered along the BRCA2 protein between positions 2336 and 2729 inclusive (394 residues, ~11% of the total). The range test was highly significant ($p = 0.0008$ and 0.0009, respectively using uniform and BIC-derived null distributions).

The twenty FA-associated truncation or frameshift mutations were distributed uniformly across the BRCA2 protein (Figure 2) ($p = 0.613$, exact Pearson statistic), with no evidence of clustering ($p = 0.312$, uniform range statistic).

Discussion

The functional effects of all of the *FANCD1/BRCA2* mutations need to be examined more closely, in order to determine whether those with two mutant alleles are truly “FA” or “variant FA”, and to provide appropriate genetic counseling, risk assessment, and cancer surveillance to relatives from both sides of the family. Our analyses suggest that FA-D1 is indeed phenotypically distinctive among the FA complementation groups, despite the known heterogeneity within FA itself. Focusing on the specific types of mutations in *BRCA2* may be instructive with regard to how patients with FA-D1 differ from patients with other types of FA, and with regard to why biallelic mutations in a breast/ovarian cancer susceptibility gene give rise to a totally different syndrome from that seen in heterozygotes.

To address this question more definitively, it is important to note that two of the patients classified as *FANCD1/BRCA2* actually had only one deleterious *BRCA2* allele, plus a benign polymorphism in the *trans* allele; they should be included in defining a new disorder with caution. In patient HSC230, the K3326X allele removes the last 92 amino acids at the carboxy-terminus, does not change the function of the gene product, is a polymorphism found in >1% of normal individuals, and is frequently found in linkage disequilibrium with a known deleterious mutation [29, 32]. In addition, this patient clearly belongs to the FA-B complementation group, with a defined deleterious X-linked mutation in *FANCB* [16]. The other benign polymorphism patient, 900/1, has N372H, also lacks known functional consequence, and is not associated with increased cancer risk [31, 32]. The report of very early-onset breast cancer in the grandmother on this side of the family, absent direct information that she carries this allele, is provocative but not informative. Unless a truly deleterious mutation is found to be in linkage disequilibrium with the N372H allele, it is possible that patient 900/1 belongs to a different complementation group than FA-D1, or even has another disorder. However, it must be emphasized that the usual methods for sequencing *BRCA2* might miss large deletions or rearrangements, which might be in *cis* to the benign mutations reported in these two patients [33]. Both patients carrying "benign" allele had physical features consistent with FA, as well as increased chromosome breakage, but 900/1 had T-cell ALL (not the typical AML of FA) at age 5, while HSC230 had no cancer by age 2.

Six patients from five families with one deleterious and one missense *BRCA2* mutation, the latter of unknown clinical significance with regard to familial breast cancer, are potentially very informative. Family histories of cancer were provided for only two of the five families, and neither was enriched with *BRCA2*-related cancers. However, it is striking that the five FA-associated missense mutations clustered tightly between amino acid positions 2336 and 2729, while the 20 truncation/frameshift mutations were uniformly distributed across the *BRCA2* gene. This "FA cluster" is located in the most highly-conserved *BRCA2* region in an inter-species comparison of human/mouse/chicken *BRCA2*, suggesting that these residues may be functionally constrained (data not shown). *BRCA2* regions which are not implicated in breast/ovarian cancer pathogenesis in heterozygous adults, nonetheless may be critical interaction regions for a component of the FA pathway, such as downstream of ubiquitination of *FANCD2* [7, 34]. This hypothesis provides a rationale for further evaluation of interactions between the BRCA2 protein and other proteins in the FA DNA repair pathway. For example, these missense mutations (hypomorphs) may reduce the amount of functional BRCA2 protein.

Alternatively, at least some of the five families with an allele of unknown significance may be truly heterozygous for a single biologically active *BRCA2* mutation; their FA could be due to unrecognized mutations in a different FA gene, as we have illustrated with HSC230, who was reported to be an example of biallelic mutations in *BRCA2* but who is clearly an FA-B patient.

In 19 of the 27 patients, and 13 of the 20 families, both alleles were interpreted to be deleterious or probably deleterious; patient 4 was a compound heterozygote for two different null mutations. No patient was homozygous for the same null allele, and thus the data on live-born children with biallelic mutations in *BRCA2* is insufficient to address the possibility that true homozygosity for mutations in *BRCA2* is incompatible with life. Survival of homozygous or compound heterozygous mice with *BRCA2* mutations seems dependent upon mutation type and

mouse strain [35, 36]. It would be of interest to determine whether mothers of FA-D1 patients experience excessive fetal loss.

Are there genuine genotype/phenotype associations among patients who do appear to have *bona fide* deleterious mutations of both *BRCA2* alleles? Short stature, microcephaly, abnormal thumbs, hyperpigmentation and/or café-au-lait spots, and renal and gastrointestinal (GI) anomalies were reported frequently, and short stature, microcephaly, and GI anomalies were more common in the FA-D1 group than in other patients with FA [1]. In the general population, the VATER association is >50 times more common than FA (16 per 1,000,000 *versus* 2.6/1,000,000 live births, respectively). It has been suggested that 5% of children in the VATER category may have FA [37], and that 5% of patients with FA have features consistent with the VATER association [25]. The identification of five cases with VATER features among 27 patients (19%) in this series suggests that there may be a higher proportion of VATER association among FA-D1 than FA overall ($p = 0.01$). However, the numbers are very small, and a VATER phenotype has also been reported in FA-A, C, E, F, and G. There may be specific *BRCA2* mutations that are linked with VATER association in the absence of a clinical diagnosis of FA.

The risk of early-onset of specific cancers is clear and dramatic in the FA-D1 group, in which the cumulative probability of leukemia was 79% by age 10, of a solid tumor was 67% by age 5, of a brain tumor was 64% by age 5, of a Wilms tumor was 64% by age 6.7 (data not shown), and of any malignancy was 97% by age 5.2 years. These probabilities are much higher than we observed in genetically-unclassified FA patients, and in our literature review [27, 38], in which the cumulative probability of leukemia reached a plateau of ~30% by age 30, and of a solid tumor 25% by age 30 and 75% by age 45 years. Thus, FA-D1 patients have a distinctively higher risk of specific cancers than patients in other FA complementation groups. In addition, the correlations between IVS7 with AML and both 886delGT and 6174delT with brain tumors suggest the possibility of specific genotype/cancer associations, although the numbers of cases and families reported to date are too small to draw firm conclusions.

The recent observation that all FA groups, including FA-J, but *excluding* FA-D1, develop nuclear Rad51 foci after DNA damage [30] underscores FA-D1's distinctiveness. The etiologic role of *BRCA2* mutations *vis-à-vis* the typical FA birth defects, or the specific cancers reported in these patients remains to be elucidated, although the associations with AML and brain tumors described above may be mutation-specific. Greater attention must be paid to the biological consequences of the specific *BRCA2* mutations identified in this context, particularly since, by conventional criteria, several appear to be without known functional consequences. How do alleles the importance of which would be dismissed in hereditary breast/ovarian cancer (HBOC) act to cause the unique phenotype observed in FA-D1 patients? Is there a protective interaction between a BRCA2 protein with a missense mutation and the normal BRCA2 protein, which is lost in patients with a truncated protein due to a deleterious mutation? Or might the recognition that some FA-D1 patients have one missense mutation imply that such mutations, heretofore of "unknown" significance are, in fact, biologically important?

A larger, more systematically-ascertained series of FA-D1 patients must be analyzed to determine whether the severe clinical phenotype observed among the limited number of patients

reported to date is truly characteristic of this FA subgroup, or whether this represents biased ascertainment or reporting of patients with more severe disease. Most important, we must determine whether single copies of the presumably deleterious *BRCA2* mutations detected in FA-D1 patients also predispose to the HBOC spectrum of cancer in heterozygous family members. Counseling those individuals is particularly difficult at present, since they are ascertained as FA family members, rather than as HBOC kindred members. In addition, some of them have altered *BRCA2* alleles that are currently of unknown clinical significance. The converse clinical presentation is also important: should women of child-bearing age from *BRCA2*-positive HBOC families be counseled regarding the theoretical risk of FA in their offspring if their partners carried unrecognized truncating or even missense *BRCA2* mutations? Such events are within the realm of possibility, particularly in specific population groups that have a higher prevalence of *BRCA2* mutations (e.g. 1% of unselected individuals of Ashkenazi Jewish descent carry the 6174delT founder mutation). The rarity of FA-D1 (~3% of all FA) suggests that a registry targeting FA-D1 patients may be the most efficient means to address the many unanswered questions created by the unexpected nexus between the FA and BRCA pathways.

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Figure Legends

Figure 1: Solid tumors in patients with FA due to mutations in *FANCD1/BRCA2*. A) Probability of any solid tumor. B) Probability of a brain tumor. C) Probability of a brain tumor according to the presence or absence of the 886delGT mutation. Blue dashed line: mutations other than 886delGT (N = 22); red solid line: 886delGT mutation (N = 5). D) Probability of any malignancy. The plateaus in all of the figures are due to patient HSC62, who reached age 30 at last report. This patient was homozygous for IVS19-1G>A, which results in an in frame deletion of exon 19, an allele for which severity is inferred but not proven.

Figure 2: Schematic of the BRCA2 protein coding region. The location of each exon is shown (numbered boxes). The location of mutant alleles found in FA patients is indicated. Orange circles: deleterious (frameshift/nonsense mutations leading to truncation; blue squares: unknown (missense mutations); green arrows: benign (not associated with cancer risk). The classification of the mutant alleles is defined in Methods.

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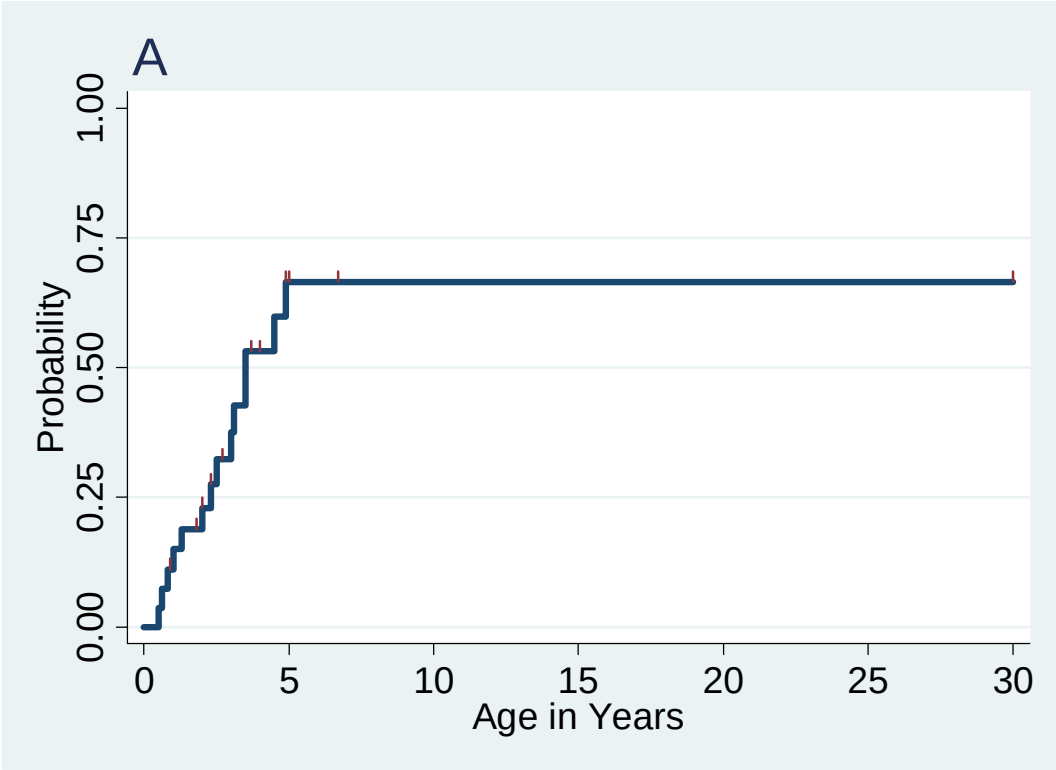

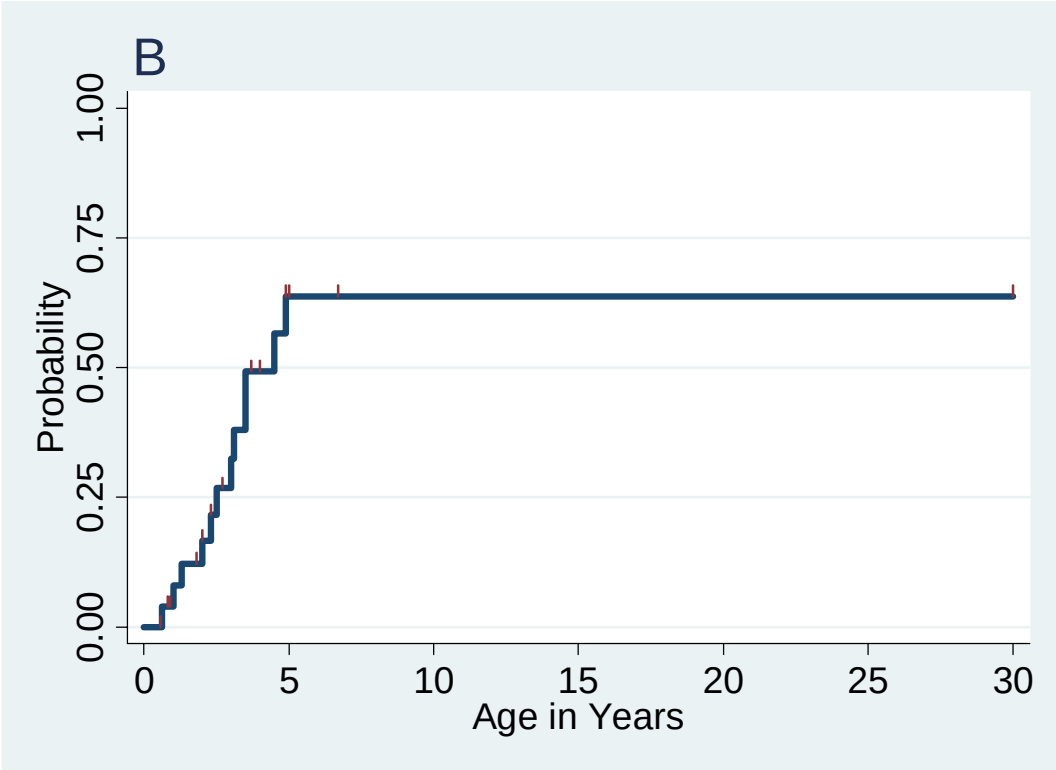

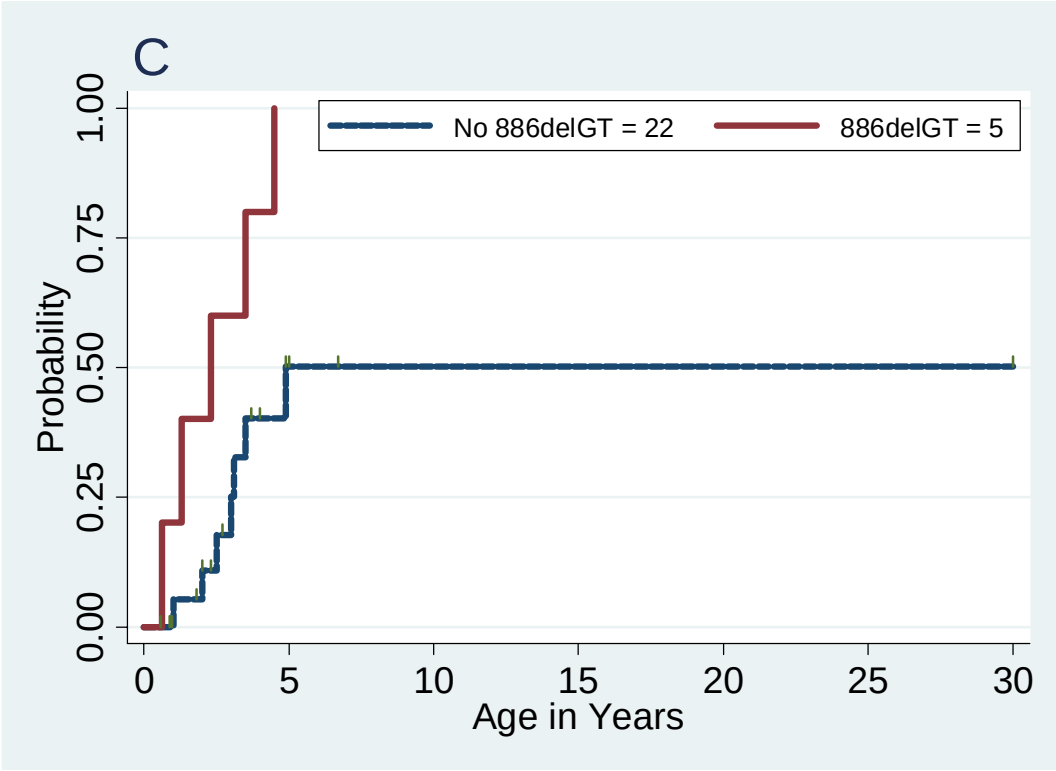

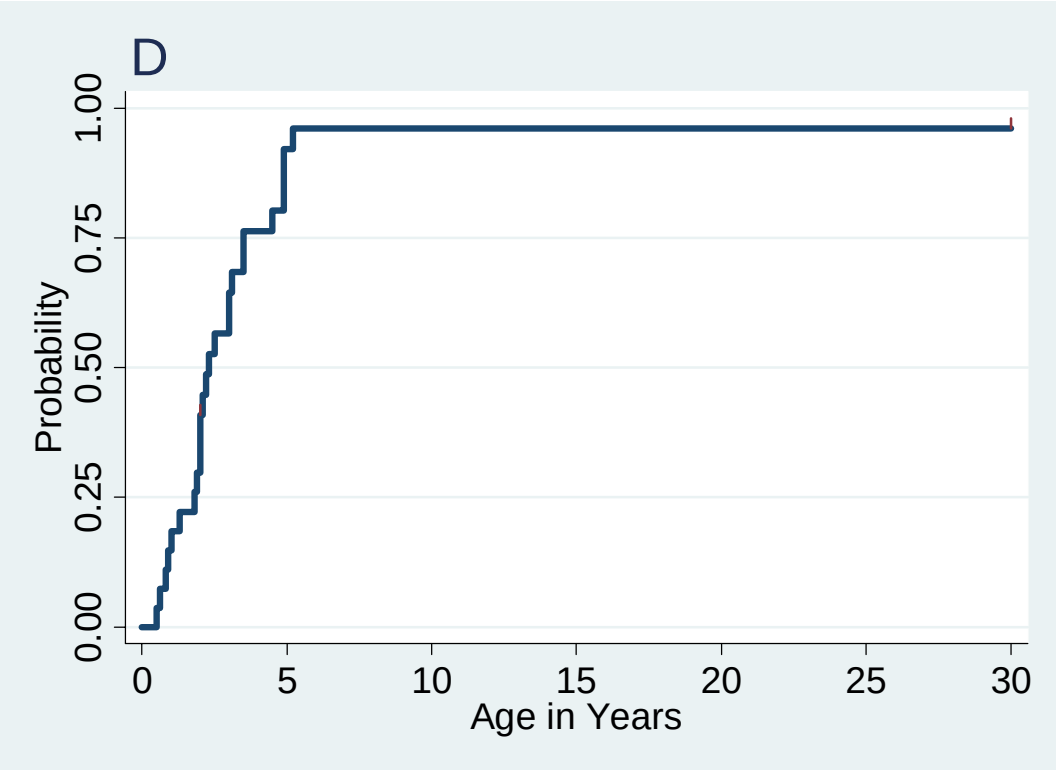

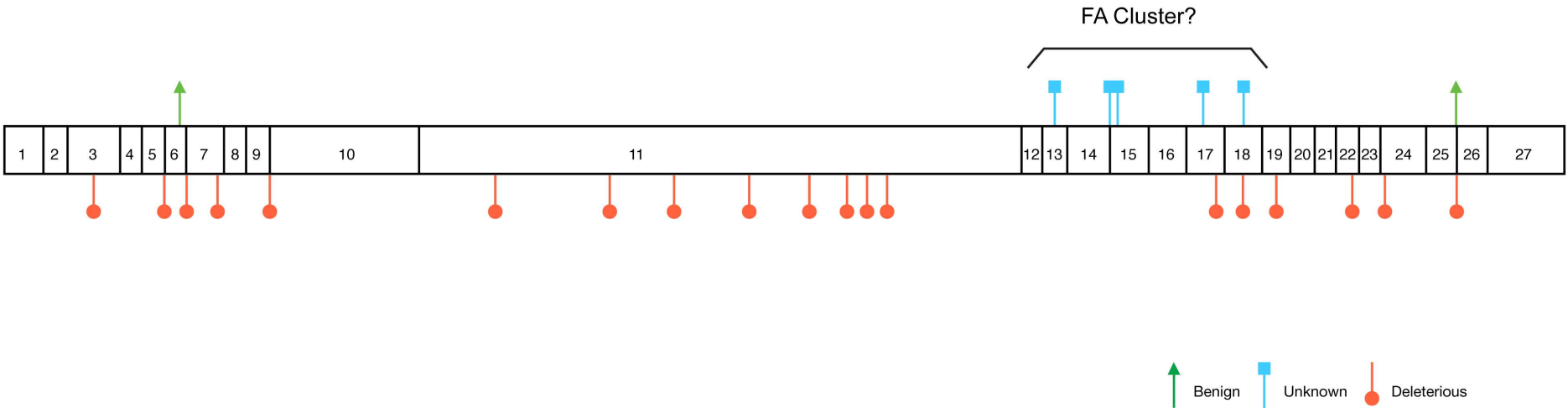
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